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D. Irwin · T. D. Leathers · R. V. Greene · D. B. Wilson

Corn fiber hydrolysis by *Thermobifida fusca* extracellular enzymes

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Abstract *Thermobifida fusca* was grown on cellulose (Solka-Floc), xylan or corn fiber and the supernatant extracellular enzymes were concentrated. SDS gels showed markedly different protein patterns for the three different carbon sources. Activity assays on a variety of synthetic and natural substrates showed major differences in the concentrated extracellular enzyme activities. These crude enzyme preparations were used to hydrolyze corn fiber, a low-value biomass byproduct of the wet milling of corn. Approximately 180 mg of reducing sugar were produced per gram of untreated corn fiber. When corn fiber was pretreated with alkaline hydrogen peroxide, up to 429 mg of reducing sugars were released per gram of corn fiber. Saccharification was enhanced by the addition of β-glucosidase or by the addition of a crude xylanase preparation from *Aureobasidium* sp.

Introduction

Over the past decade, considerable progress has been made in the study of glycoside hydrolases. Over 60 sequence-based enzyme families are now known and many hundreds of structural genes have been cloned, expressed and characterized (http://afmb.cnrs-mrs.fr/CAZY). Our understanding of the activity and binding of these enzymes on different defined substrates has improved and mutation studies have elucidated the mechanisms of hydrolysis by individual enzymes. However, many questions remain to be answered about the degradation of complex biomass substrates. Corn fiber, a byproduct of the wet milling process, has been targeted as a substrate of particular interest, because of its abundance, ready availability and low value (Leathers 1998). It is estimated that ethanol yields from corn could be increased by approximately 10% if the constituent sugars of corn fiber could be efficiently utilized (Gulati et al. 1996). Corn fiber is primarily composed of the outer seed covering or pericarp of the kernel, along with adherent starch (May 1987). Its apparent composition varies considerably according to its source and the method of analysis (Leathers 1998). Our corn fiber samples contained approximately 20% xylose and 10% arabinose in the form of arabinoxylan, 24% cellulose and 20% adherent starch (Leathers and Gupta 1996). Although corn fiber is a potentially rich source of fermentable sugars, it is recalcitrant to enzymatic digestion. In particular, corn fiber arabinoxylan appears to be highly substituted and resistant to saccharification (Hespell 1998; Hespell et al. 1997; Saulnier et al. 1995). Partial hydrolysis of corn fiber xylan has been achieved using crude enzymes from the yeastlike fungus *Aureobasidium* sp. (Leathers and Gupta 1996).

Thermobifida fusca (formerly *Thermomonospora fusca*) is a thermophilic actinomycete that grows well on carbohydrate polymers, such as cellulose, starch or xylan. Six cellulase genes and one xylanase gene have been cloned and expressed from *T. fusca* (Irwin et al. 1993, 1994, 2000). Recently, the genome of this organism was sequenced, resulting in a wealth of new information about the encoded enzymes (http://genome.ornl.gov/microbial/tfus/). It is relatively easy to clone individual enzymes from *T. fusca* and express them in *Escherichia coli* or *Streptomyces lividans*, which makes detailed studies of enzyme structure and function possible. Thermophilic enzymes are attractive for large-scale applications, be-

D. Irwin · D. B. Wilson (✉)
Department of Molecular Biology and Genetics,
Cornell University,
458 Biotechnology Building, Ithica, NY 14850 USA
e-mail: dbw3@cornell.edu
Tel.: +1-607-2555706
Fax: +1-607-2552428

T. D. Leathers
Fermentation Biotechnology Research Unit,
National Center for Agricultural Utilization Research,
Agricultural Research Service,
United States Department of Agriculture,
Peoria, IL 61604 USA

R. V. Greene
Office of International Programs, Agricultural Research Service,
United States Department of Agriculture,
Beltsville, MD 20705 USA

cause they have the potential to retain activity at higher temperatures during prolonged reactions. However, *T. fusca* does not produce the quantity of cellulases that are produced by fungal organisms such as *Trichoderma reesei* (2–4 g extracellular protein/l; Shoemaker et al. 1981). In this work, we tested crude concentrated supernatant enzymes from *T. fusca* ER1 fermentor cultures grown on corn fiber (TFC), xylan (TFX), or Solka-Floc cellulose (TFSF) for their ability to saccharify untreated, boiled, or alkaline hydrogen peroxide (AHP)-pretreated corn fiber. We also tested combinations of these enzymes with each other and with a crude xylanase preparation from *Aureobasidium* sp. (AX).

Materials and methods

Corn fiber, AHP-pretreated corn fiber and AX were prepared as described by Gould (1984) and Leathers and Gupta (1996). The β-glucosidase used was BglC, cloned from *T. fusca* and purified from *E. coli* (Spiridonov and Wilson 2001).

Preparation of concentrated crude supernatants

A 250-ml culture of *T. fusca* ER1, a *T. fusca* YX low-protease mutant, was grown overnight at 50 °C in Hägerdahl minimal medium (Hägerdahl et al. 1978) plus 0.2% cellobiose. This culture was used to inoculate a 10-l (working volume) fermentor containing Hägerdahl minimal medium plus 100 g of corn fiber. This medium was prepared by autoclaving the water and macro salts together with the untreated corn fiber and adding the micro salts, biotin and thiamine as sterile solutions. The fermentor growth conditions were pH 7.3, 50 °C, dissolved oxygen greater than 40%, stirring at 200 rpm and airflow at 14 l/min. The culture was harvested after 28 h and centrifuged at 3,590 *g* for 30 min, to remove the remaining corn fiber and cells. The supernatant was pumped through a Pellicon 0.22-μm cassette filter unit (Millipore, Bedford, Mass.) until only 1 l remained in the recycled fraction that not pass through the membrane. Of the original activity, 40% was found in the filtered supernatant but, after concentration, less than 10% of the activity was recovered. Approximately 30% of the original enzyme activity was found in the concentrated unfiltered material. This material was clarified by centrifugation at 16,000 *g* for 30 min and was used as TFC for the experiments in this paper. Supernatants were collected from cultures grown similarly on larchwood xylan (ICN, Costa Mesa, Calif.) or cellulose (Solka-Floc, James River Corp., Berlin, N.H.) as sole carbon sources. Glycerol was added to these supernatants to 10% and they were successfully filtered through the 0.22 μm filter and concentrated using a Pellicon 10,000 MWCO cassette. Larchwood xylan- and cellulose-grown concentrates were labeled TFX1 and TFSF, respectively. TFX2 was prepared similarly from a 200-ml culture of *T. fusca* grown on birchwood xylan (Sigma X-0502). Concentrated supernatants were stored at −70 °C. ATCC accession numbers are BAA-629 for *T. fusca* YX and BAA-630 for *T. fusca* ER1.

Assay methods

Protein concentrations of concentrated supernatants and cell extracts were determined by the Bradford method (Bradford 1976), using bovine serum albumin as a standard. Cell pellets were resuspended in water at one-half their original volume and disrupted in a French press at 10,000 psi. Cellular debris was removed by centrifugation and cytoplasmic protein was determined as above. Protein concentrations of unconcentrated supernatants were measured by the TCA Lowry protein assay (Lowry et al. 1951).

Enzyme assays were performed at 50 °C in 0.05 M sodium acetate buffer at pH 5.5, with 0.02% sodium azide added to prevent microbial growth, unless otherwise noted. Assays measuring the hydrolysis of 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-xylopyranoside, 4-nitrophenyl α-D-xylopyanoside, 4-nitrophenyl β-D-cellobioside, or 4-nitrophenyl α-D-arabinofuranoside (all from Sigma Chemical Co., St. Louis, Mo.) were performed in 0.1 ml, using 2.5 mM substrate for 30–60 min. The reactions were stopped by the addition of 0.9 ml of 1.0 M Na_2CO_3 and absorbances were read at 400 nm. The molar extinction coefficient of 4-nitrophenol at this wavelength and pH is 1.83×10^4/cm.

Activity assays using low viscosity carboxymethyl-cellulose (CMC, 10 mg/ml; Sigma), phosphoric acid-swollen cellulose (SC, 2.5 mg/ml; swollen Sigma cell 100; Ferchak et al. 1980), filter paper (FP, 8 mg/ml; Whatman no. 1) and xylan (XY, 5 mg/ml; Sigma birchwood xylan) were performed as described by Irwin et al. (1993), using the dinitrosalicylic acid (DNS) method to quantitate total reducing sugars (Ghose 1987). Due to the heterogeneity of the complex natural substrates and the nonlinearity of the reactions, specific activities were calculated at an arbitrary percent digestion of 5%, except for SC which was calculated at 10% digestion. Glucose was determined using a YSI Biochemistry Analyzer fitted with glucose and ethanol sensors. HPLC analysis of glucose, arabinose and xylose was carried out as described by Leathers and Gupta (1996). Corn fiber digestion assays were run using 10 mg of corn fiber in 1 ml of 0.05 M sodium acetate buffer at pH 5.5 with 0.02% sodium azide. Tubes were rotated end-over-end at the indicated temperatures and centrifuged at the desired times. Then, 30 μl portions of the supernatant were removed in triplicate and reducing sugars were measured by the DNS method (Ghose 1987). Total reducing sugars were estimated using a standard curve for glucose, which somewhat underestimated the actual amount of sugars.

Thin layer chromatography of hydrolysis products was performed using Whatman LK5D 150-A silica gel thin layer plates with ethyl acetate:water:MeOH (40:15:20) as the separating solvent. Plates were stained (100 ml of acetic acid, 1 ml of *p*-anisaldehyde, 2 ml of concentrated sulfuric acid) and then heated for 1 h at 95 °C, as described by Chirico and Brown (1985) and Jung et al. (1993). Glucose and xylose oligomer standards were obtained from Sigma.

Results

T. fusca was able to grow well in minimal medium with corn fiber, Solka-Floc, or xylan as the sole carbon source. A set of *T. fusca* ER1 shake-flask cultures grown on glucose, cellobiose, xylan, Solka Floc and corn fiber had 336, 399, 481, 422 and 153 mg protein/l in the cell extracts and 22, 63, 115, 187 and 92 mg protein/l in the supernatants. The ratio of supernatant to cellular protein ranged from 0.065 for glucose to 0.604 for corn fiber. The yields of protein from the harvested and concentrated fermentor supernatants used in this study were 95, 170 and 92 mg/l for TFC, TFSF and TFX1, respectively. Only about 30% of the filter paper and xylanase activities were recovered from the corn fiber-grown fermentor, due to the inability of the supernatant to pass through the 0.22-μm filter cassette, as noted in the Materials and methods section. SDS-PAGE (Fig. 1a) showed that the protein pattern of TFC was similar to that of the original supernatant. The cell-extract protein concentration of the corn fiber fermentor at harvest was 457 mg/l of

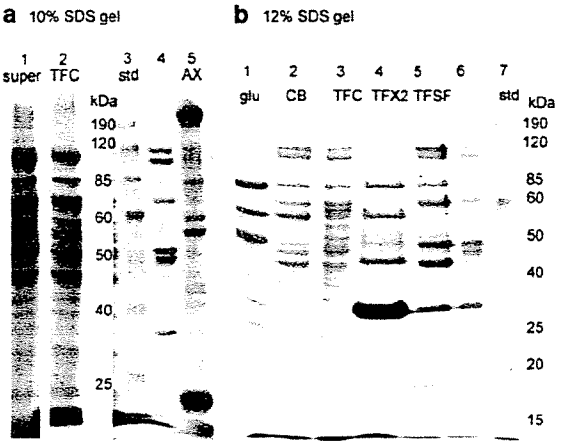

Fig. 1a, b SDS-PAGE of supernatant proteins. See Materials and methods for abbreviations. **a** *Lane 1* Lyophilized corn fiber-grown supernatant before filtration, *lane 2* 31 µg TFC, *lane 4* 1 µg of each of the cloned and purified *Thermobifida fusca* proteins [Cel48A (104 kDa), Cel9A (90 kDa), Cel6B (60 kDa), Cel5A (46 kDa), Cel6A (43 kDa), TFX11A (32 kDa)], *lane 5* 2.4 µg of *Aureobasium* xylanase. **b** Concentrated supernatants from cultures of *T. fusca* grown on various carbon sources: *lane 1* glucose (5 µg), *lane 2* cellobiose (5 µg), *lane 3* corn fiber (10 µg), *lane 4* birchwood xylan (5 µg), *lane 5* Solka-Floc (10 µg), *lane 6* same as Fig. 2a, lane 4. Protein standards (*std*) were from Life Technologies, Gaithersburg, Md.

culture, showing much better growth than in the shake-flask experiment above.

The wide variety of proteins that this organism is capable of secreting in response to different carbon sources is illustrated in Fig. 1b. The purified cellulase bands shown in lane 6 are present in Solka-Floc- and cellobiose- but not glucose- or xylan-grown cultures. The 32-kDa xylanase, TFX11A, was prominent in TFX1 (data not shown) and TFX2 preparations and was also present in TFSF but was faint in TFC. The TFC protein pattern showed a more complex set of bands of similar intensities. The AX preparation is shown in Fig. 1a, exhibiting intense bands at about 20 kDa and 200 kDa.

The activities of the concentrated supernatants are summarized in Table 1. The TFC and TFX preparations included significant levels of amylase. Corn fiber contains adherent starch, but it is not clear why TFX contained amylase activity. In contrast, TFSF included little or no amylase. TFC exhibited cellulase activities (CMC, FP, SC) at a level between those of TFSF and TFX; but the xylanase activity was low, which suggests that *T. fusca* was unable to attack the xylan component of corn fiber. AX had high xylanase activity (438 µmol of reducing sugar/mg of protein per minute) at 37 °C. The CMC and amylase activities of AX were previously reported to be less than 0.01% of the xylanase activity (Leathers and Gupta 1996).

The digestion of corn fiber by the various enzyme preparations is shown in Fig. 2. As normalized by total protein added, all of the *T. fusca* enzyme sources had limited activity against untreated or boiled corn fiber,

Table 1 Activities of concentrated supernatants from cultures grown on different carbon sources at 50 °C

Culture carbon source	Product (mg protein/l supernatant)	Activity 1 [µmol *p*-nitrophenol (pNP)/mg protein per minute)][a]					Activity 2 (µmol reducing sugar/mg protein per minute)				
		pNP β-D-glucose	pNP β-D-xylose	pNP α-D-xylose	pNP β-D-cellobiose	pNP-α-arabinose	CMC[b]	SC[b]	FP[c]	Amylase[d]	XY[e]
Corn fiber (TFC)	232	0.157	0.012	0.005	0.072	0.013	2.27	1.67	0.05	0.229	4
Xylan (TFX1)	91	0.065	0.074	–	0.200	0.024	0.59	<0.04	<0.015	0.306	175
Xylan (TFX2)	115	0.014	0.055	0	4.090	0.019	1.34	0.21	–	0.471	760
Solka floc (TFSF)	351	0.033	0.054	0	0.780	0.038	7.88	6.58	0.21	<0.0155	76

[a] pNp (2.5 mM substrate) assays were done for 30 min
[b] Low-viscosity carboxy methylcellulase (CMC, 10 mg/ml; Sigma) and phosphoric acid-swollen cellulose (SC, 2.5 mg/ml) assays were done for 2 h and activities were calculated at 5% and 10% digestion, respectively
[c] Filter paper (FP, 8.5 mg/ml) assays were done for 16 h and activities were calculated at 5% digestion
[d] Amylase (soluble starch, 5 mg/ml; Mallinkrodt) assays were done for 16 h and activities were calculated at 5% digestion
[e] Birchwood xylan (XY, 5 mg/ml; Sigma) assays were done for 15 min and activities were calculated at 5% digestion

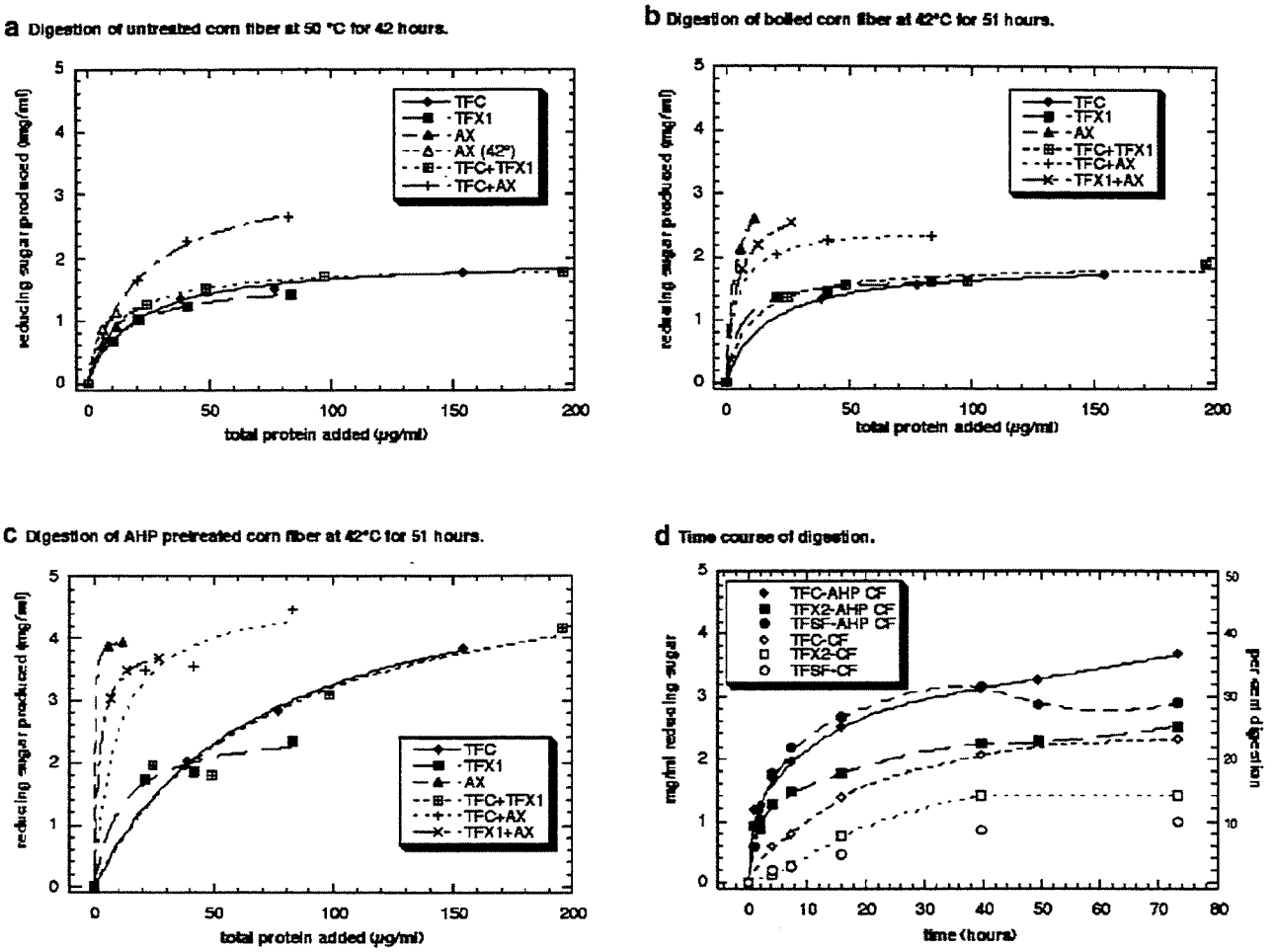

Fig. 2 Hydrolysis assays of 10 mg corn fiber/ml: **a** untreated, **b** boiled and **c** alkaline hydrogen peroxide (AHP)-pretreated, using varying amounts of TFC, TFX1, AX and mixtures of these enzymes at the following milligram ratios: *TFC+TFX1* 3.7:1.0, *TFC+AX* 12.8:1.0, *TFX1+AX* 3.5:1.0. **d** Time-course of the hydrolysis of corn fiber and AHP-pretreated corn fiber by TFC, TFSF and TFX2. Time-course assays were run at 50 °C, using 100 µg protein/ml; and an additional 3 µg of *T. fusca* β-glucosidase/ml was added to the AHP-pretreated corn fiber assays

with the maximum digestion reaching only about 16% (Fig. 2a, b). AHP pretreatment significantly increased the extent of hydrolysis by TFC and TFX and 40% hydrolysis was achieved by a combination of these two preparations (Fig. 2c). The activity of TFX on AHP-pretreated corn fiber was greater than that of TFC at lower enzyme concentrations but reached a maximum value at 20% digestion. Time-course experiments (Fig. 2d) revealed that more than half of the hydrolysis of AHP-pretreated corn fiber took place in the first 4 h of digestion, while the hydrolysis of untreated corn fiber was more gradual. TFSF was nearly as active as TFC for the first 40 h of hydrolysis on AHP-treated corn fiber, although the extent of hydrolysis by TFSF appeared to level off at 30–33%, while that of TFC was still increasing after 72 h.

AX had a markedly increased reducing sugar production on boiled corn fiber over untreated corn fiber and AHP-pretreatment of the corn fiber allowed a further increase, to 40% hydrolysis by a small amount of AX. On untreated corn fiber, TFC plus AX had an increased activity, although not higher than AX alone on boiled corn fiber. TFC plus AX increased the activity on AHP corn fiber over TFC alone and reached 43% hydrolysis. Based on the measured activities (Table 1), TFC may contribute cellulase and amylase to the xylanase in AX.

The hydrolysis products of AHP corn fiber digestions were analyzed by thin layer chromatography (Fig. 3). In this method, six carbon sugar bands were green, while five carbon sugars appeared as relatively lighter brown bands. TFC digestion of AHP corn fiber produced increasingly darker bands with time, corresponding to G1–G3 in agreement with the increased reducing sugar production.TFX2 digestion of corn fiber generated a pattern of brown bands after 4 h that did not change further with time. TFX2 digestion of AHP corn fiber was similar, but appeared more green in color; and the G2 band appeared to increase with time. TFSF showed markedly increased G1–G3 products on AHP corn fiber,

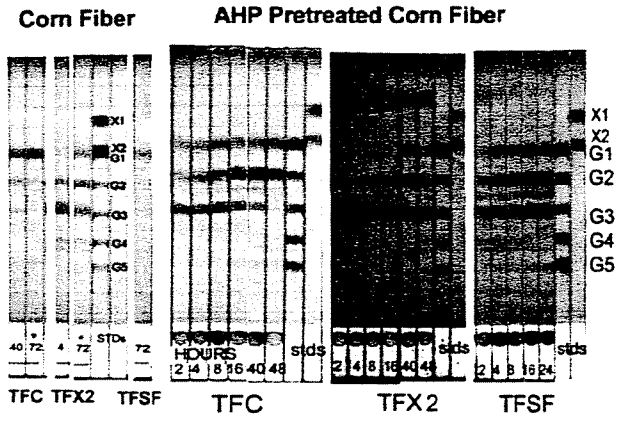

Fig. 3 Thin layer chromatography showing a time-course of the products of hydrolysis of corn fiber and AHP-pretreated corn fiber by 100 μg of TFC, TFX2, or TFSF. Standards: *X1* xylose, *X2* xylobiose (20 μg of each) *G1–G5* Glucose-cellopentaose (5 μg of each). The hours of hydrolysis are noted in each lane

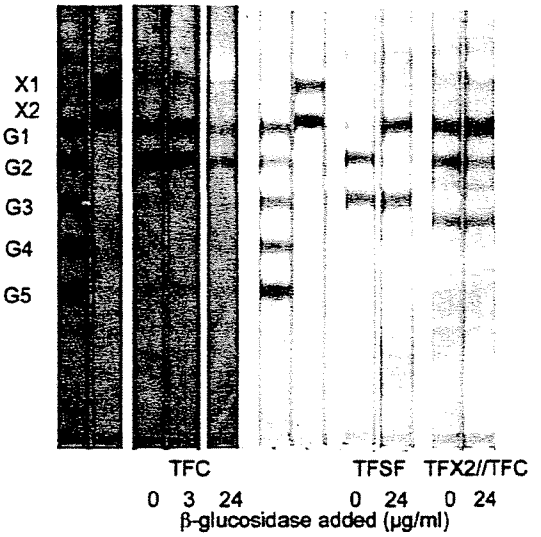

Fig. 4 Effect of added β-glucosidase on hydrolysis products from digestion of 10 mg of AHP-pretreated corn fiber/ml. The enzymes used were TFC (100 μg, 53 h), TFSF (100 μg, 53 h) and TFX2/TFC (100 μg TFX2 for 4 h, followed by 100 μg TFC for 53 h). Standards were as in Fig. 3

as opposed to untreated corn fiber. AX produced G1, X2 and a fainter X1 band, while a combination of TFC and AX produced both a strong G1 band and fainter X1 and X2 bands (data not shown).

The addition of BglC to TFSF digests of AHP corn fiber converted most of the G2 to G1, although a strong G3 band remained (Fig. 4). In contrast, the addition of β-glucosidase to TFC digests converted only a portion of the G2 band to glucose and there was no G3 band in the product mixture. Since the BglC enzyme activity was not inhibited by TFC preparations (data not shown), it is likely that the G2 band in TFC digests contained material other than cellobiose. Glucose and DNS assays (Table 2) confirmed that the addition of BglC increased the levels of free glucose from digests of AHP corn fiber. Digestion of AHP corn fiber with TFX2 for 4 h, followed by TFC digestion for an additional 53 h, produced the highest yield: 213 mg glucose/g and 429 mg of total reducing sugar/g.

HPLC analysis of glucose, xylose and arabinose in 47–51 h hydrolysates showed that only AX produced arabinose (Table 3). TFC or TFSF produced only glucose, at about one-quarter of the level of total reducing sugars. TFX1 preparations produced little free xylose, even from AHP-pretreated corn fiber. Overall, HPLC measurements were consistent with the TLC observations that much of the solubilized reducing sugars were oligosaccharides. The absence of arabinose in the products from TFC or TFX1 digestions combined with the low pNP α-arabinose activity of the supernatants indicates that *T. fusca* supernatant enzymes are not able to break down products containing arabinose.

Discussion

This study produced some intriguing results. It is unclear why the supernatant enzymes could not pass through a 0.22-μm filter, which is normally used for filtering out bacterial cells. Possibly the enzymes were bound to material that was too large to pass through the filter but not dense enough to be removed by centrifugation.

Table 2 Effect of β-glucosidase on hydrolysis of alkaline hydrogen peroxide (AHP)-treated corn fiber. For abbreviations, see Table 1

β-Glucosidase added (μg/ml)	TFC (100 μg/ml, 53 h)		TFSF (100 μg/ml, 53 h)		TFX2 (100 μg/ml, 4 h) followed by TFC (100 μg/ml, 53 h)	
	Glucose[a] (mg/ml)	Total reducing sugars[b] (mg/ml)	Glucose[a] (mg/ml)	Total reducing sugars[b] (mg/ml)	Glucose[a] (mg/ml)	Total reducing sugars[b] (mg/ml)
0	0.62	3.66	0.20	3.39	1.58	4.05
3	1.72	3.94	1.24	3.49	–	–
6	1.18	4.11	1.44	3.45	–	–
12	1.03	3.91	1.71	3.63	–	–
18	1.90	4.08	1.76	3.77	–	–
24	1.43	3.91	1.80	3.81	2.13	4.29

[a] Glucose was determined by a YSI Biochemistry Analyzer fitted with glucose and ethanol sensors. No ethanol was detected in any sample. [b] Total reducing sugars was determined by the DNS method (see Materials and methods).

Table 3 HPLC identification of monosaccharide hydrolysis products. *cf* Corn fiber

Sample	Substrate	Temperature (°C)	µg protein/ml in assay	Glucose (mg/ml)	Xylose (mg/ml)	Arabinose (mg/ml)	Total reducing sugar (DNS)
TFC (51 h)	Boiled cf	50	154	1.002	0	0	1.71
TFX1 (51 h)	Boiled cf	50	83	0.673	0	0	1.62
TFC+TFX (51 h)	Boiled cf	50	154+42	1.18	0.052	0	1.86
TFC+AX (51 h)	Boiled cf	50	77+6	1.69	0.182	0.759	2.32
TFX1+AX (51 h)	Boiled cf	50	21+6	1.77	0.193	0.716	2.53
AX (48 h)	Boiled cf	44	2.4	1.48	0.078	0.6	2.1
TFC (47 h)	AHP cf	50	50	0.446	0	0	2.8
TFX1 (47 h)	AHP cf	50	50	0.514	0.066	0	2.8
TFC+TFX1 (47 h)	AHP cf	50	25+25	0.375	0	0	2.5
AX (48 h)	AHP cf	44	2.4	2.0	0.127	0.855	3.1

Possibly, the enzymes are in non-cell-associated cellulosome-like complexes which are produced by growth on corn fiber but not on Solka-Floc. Cell-associated cellulosome-like structures were seen for *T. fusca* TM51 grown on cellulose but not on glucose (Kukolya et al. 2001) and for *T. curvata* grown on cellulose, xylan or cellobiose (Bonner and Stutzenberger 1988; Hostalka et al. 1992; Stutzenberger 1991).

Clearly, *T. fusca* is able to respond to the challenge of biomass substrates by increasing the quantity and variety of enzymes produced. BglC activity was previously thought to be present only in the cell-associated material. However, growth on corn fiber produced a five-fold increase in supernatant BglC activity over that in Solka-Floc-grown cultures. This is comparable with the result that *Thermomonospora curvata* produced 16 times more BglC when grown on protein-extracted lucerne fiber, compared with cellulose (Bernier and Stutzenberger 1988). Significant amylase activity was produced in the xylan- and cornfiber-grown supernatants but not in Solka-Floc supernatants. This is consistent with a study which showed that, in the presence of cellulose or cellobiose, the uptake of the amylase inducer, maltose, is competitively blocked by cellobiose (Busch and Stutzenberger 1997). TFX, but not TFC, could produce xylose oligomers, but not xylose or xylobiose, from corn fiber. There are open reading frames in the genome that are homologous to alpha and beta xylosidases and arabinofuranosidases, but these genes were apparently not expressed by growth on the substrates tested. TFX2 has higher pNP β-D-cellobiose, CMC and SC activity than does TFX1. Birchwood xylan may contain more cellulose than Larchwood xylan. TFSF has fairly high xylanase activity, which may be due to induction by xylan in Solka Floc. There is an enzyme in the *Thermobifida fusca* genome that is homologous to a family 10 *T. alba* endoxylanase that has the 14 base pair DNA sequence upstream that is found upstream of all purified cellulases. This sequence has been shown to be the binding site for CelR, a transcriptional regulatory protein thought to be a repressor (Spiridonov and Wilson 1999). Cellobiose binds to CelR and weakens its binding to the DNA sequence. Thus, the production of this xylanase is induced by cellobiose, as are the cellulases.

In conclusion, *T. fusca* is an attractive potential source of hydrolytic enzymes, because it is a thermophile and because the molecular biology of this system is well developed. Enzymes from *T. fusca* produced up to 18% and 43% in reducing sugar equivalents from corn fiber and AHP-pretreated corn fiber, respectively. Analysis of the hydrolysis products indicated the absence of arabinofuranosidase activity. Saccharification was enhanced by the addition of BglC or crude *Aureobasidium* sp. AX. This suggests that a complementary mixture of enzymes from diverse sources ultimately may prove most useful for utilization of this abundant biomass resource.

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